CaMKII controls the establishment of cocaine's reinforcing effects in mice and humans

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Abstract: Although addiction develops in a considerable number of regular cocaine users, molecular risk factors for cocaine dependence are still unknown. It was proposed that establishing drug use and memory formation might share molecular and anatomical pathways. Alpha-Ca(2+)/calmodulin-dependent protein kinase-II (CaMKII) is a key mediator of learning and memory also involved in drug-related plasticity. The autophosphorylation of CaMKII was shown to accelerate learning. Thus, we investigated the role of CaMKII autophosphorylation in the time course of establishing cocaine use-related behavior in mice. We found that CaMKII autophosphorylation-deficient CaMKII(T286A) mice show delayed establishment of conditioned place preference, but no changes in acute behavioral activation, sensitization or conditioned hyperlocomotion to cocaine (20 mg kg(-1), intraperitoneal). In vivo microdialysis revealed that CaMKII(T286A) mice have blunted dopamine (DA) and blocked serotonin (5-HT) responses in the nucleus accumbens (NAcc) and prefrontal cortex after acute cocaine administration (20 mg kg(-1), intraperitoneal), whereas noradrenaline responses were preserved. Under cocaine, the attenuated DA and 5-HT activation in CaMKII(T286A) mice was followed by impaired c-Fos activation in the NAcc. To translate the rodent findings to human conditions, several CAMK2A gene polymorphisms were tested regarding their risk for a fast establishment of cocaine dependence in two independent samples of regular cocaine users from Brazil (n=688) and Switzerland (n=141). A meta-analysis across both samples confirmed that CAMK2A rs3776823 TT-allele carriers display a faster transition to severe cocaine use than C-allele carriers. Together, these data suggest that CaMKII controls the speed for the establishment of cocaine's reinforcing effects.

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ORIGINAL ARTICLE

αCaMKII controls the establishment of cocaine's reinforcing effects in mice and humans

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Although addiction develops in a considerable number of regular cocaine users, molecular risk factors for cocaine dependence are still unknown. It was proposed that establishing drug use and memory formation might share molecular and anatomical pathways. Alpha-Ca^{2+}/calmodulin-dependent protein kinase-II (αCaMKII) is a key mediator of learning and memory also involved in drug-related plasticity. The autophosphorylation of αCaMKII was shown to accelerate learning. Thus, we investigated the role of αCaMKII autophosphorylation in the time course of establishing cocaine use-related behavior in mice. We found that αCaMKII autophosphorylation-deficient $αCaMKII^{T286A}$ mice show delayed establishment of conditioned place preference, but no changes in acute behavioral activation, sensitization or conditioned hyperlocomotion to cocaine (20 mg kg^{-1}, intraperitoneal). *In vivo* microdialysis revealed that $αCaMKII^{T286A}$ mice have blunted dopamine (DA) and blocked serotonin (5-HT) responses in the nucleus accumbens (NAcc) and prefrontal cortex after acute cocaine administration (20 mg kg^{-1}, intraperitoneal), whereas noradrenaline responses were preserved. Under cocaine, the attenuated DA and 5-HT activation in $αCaMKII^{T286A}$ mice was followed by impaired c-Fos activation in the NAcc. To translate the rodent findings to human conditions, several CAMK2A gene polymorphisms were tested regarding their risk for a fast establishment of cocaine dependence in two independent samples of regular cocaine users from Brazil ($n = 688$) and Switzerland ($n = 141$). A meta-analysis across both samples confirmed that CAMK2A rs3776823 TT-allele carriers display a faster transition to severe cocaine use than C-allele carriers. Together, these data suggest that αCaMKII controls the speed for the establishment of cocaine's reinforcing effects.

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INTRODUCTION

Cocaine is the second most prevalent illegal drug in the United States and Europe after Cannabis, with a lifetime prevalence among young adults of 6.3% in Europe (15–34 years),[1] 13.3% in the United States (18–25 years)[2] and 10.8% in Brazil (30–39 years).[3] The use of cocaine develops into addiction in a significant number of individuals;[4] 5–6% of cocaine users becoming cocaine dependent in the first year of use, while 15–16% establishing cocaine dependence within 10 years.[5] It was proposed that development of drug-use behavior[6] and later of addiction may be mediated by learning and memory pathways.[7] Ca^{2+}/calmodulin-dependent protein kinase-II (CaMKII) has a key role in the plasticity of glutamatergic synapses of the brain[8] and appears crucial for learning and memory, with the α-subunit composed heteromer (αCaMKII) being the most influential.[9,10] CaMKII activation contributes to the development and maintenance of drug use associated behaviors.[11–13] Cocaine exposure increases αCaMKII expression levels and αCaMKII phosphorylation at Thr286 in the striatum of rodents and humans,[14,15] which is essential for the motivation to self-administer cocaine.[16] Drug-induced reinstatement of cocaine self-administration was associated with enhanced Ca^{2+} channel stimulation, Ca^{2+} influx, CaMKII activation and phosphorylation of CaMKII at the Thr286 site, and subsequent AMPA receptor regulation.[17] A recent study identified the ventral tegmental area–nucleus accumbens (NAcc) axis as a crucial site of CaMKII action in the establishment of cocaine-induced synaptic plasticity and conditioned place preference (CPP).[18,19]

Following transient Ca^{2+}/calmodulin activation, αCaMKII can phosphorylate itself which renders the kinase autonomous. This action is known as autophosphorylation. It depends on a phosphorylation at the Thr286 site. Mice with a point mutation in position 286 of the protein ($αCaMKII^{T286A}$) do not show autophosphorylation, but severe learning impairments[20,21] and emotional dysregulation[22] suggesting that autophosphorylation controls in particular the speed of learning, but not the capacity of learning or retrieval.[23] Previous work indicates that αCaMKII autophosphorylation has an important role in the establishment of alcohol reinforcement[24] and drinking behavior in both mice and humans.[25]

Here we asked which role αCaMKII has in the rate by which cocaine use-related behavior is established in mice and humans. We hypothesized that a genetically induced deficiency in αCaMKII

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autophosphorylation results in slower establishment of drug use behavior mediated by reduced activation of mesolimbic monoaminergic activation. Furthermore, we investigated in two independent human samples whether single-nucleotide polymorphisms (SNPs) putatively impacting αCaMKII function can influence the risk for a fast transition to heavy cocaine use once consumption had commenced.

MATERIALS AND METHODS

Animals

Male and female αCaMKII^{T286A} mutant mice[20] were studied in sex-balanced designs in all experiments (overall: Mt: $n=30$; WT: $n=30$; Ht: $n=31$). This mutation blocks the autophosphorylation of CaMKII, but does not affect the Ca^{2+}-dependent activity[23] (for details: see the Supplementary Information). All housing and experimental procedures were performed in accordance with the UK Home Office Animals (Experimental Procedures) Act 1986.

CPP, sensitization and conditioned hyperactivity

Apparatus: TSE Place Preference test boxes (Bad Homburg, Germany) were used[24] (for details: see Supplementary Information). *CPP Establishment*: Cocaine-naïve animals (Mt: $n=14$; WT: $n=14$; Ht: $n=16$) were injected (intraperitoneally) before each trial with either saline or cocaine (20 mg kg^{-1}), immediately transferred (15–20 s) to the testing suite and placed into the CPP boxes. The experiment involved four phases; habituation trial (one session), conditioning trials (14 sessions) and preference tests (four sessions). Trials were performed once daily. *Habituation (d1)*: Mice were injected with saline and introduced into the center compartment with free access to all three compartments for 20 min. *Pre-test (d2)*: Mice were injected with saline and introduced into the center compartment with free access to all three compartments for 20 min. *Conditioning trials (d3-4, d6-9 and d11-18)*: Mice were conditioned using a counterbalanced design. All animals received seven pairings with saline and seven pairings with cocaine in total. Mice were injected with either saline or cocaine (20 mg kg^{-1}, intraperitoneally) and introduced into one of two compartments, with restricted access, for 20 min. *Preference tests (d5, d10, d19 and d26)*: Preference tests were performed after one, three and seven conditioning trials. Before each test, mice were injected with saline and introduced into the center compartment with free access to all three compartments for 20 min.[24] Place preference was calculated as preference change vs baseline for each individual animal. A preference score was calculated from log-transformed values to normalize skewed data.[26]

Acute locomotor effects and sensitization. Cocaine- and saline-induced locomotor activity and their sensitization were automatically scored as locomotor activity in the conditioning and pseudo-conditioning compartment after the first and seventh treatment, respectively.

Conditioned hyperactivity. Cocaine- and saline-conditioned activity was automatically scored as the locomotor activity in the conditioning and pseudo-conditioning compartment, respectively, during undrugged testing during baseline and all subsequent test trials.

In vivo microdialysis

Mice were deeply anesthetized and two guide cannulas were aimed at the prefrontal cortex (PFC) and the NAcc.[27] *In vivo* microdialysis for dopamine (DA), serotonin (5-HT) and noradrenaline (NA) was performed as described previously.[25,28] An injection of cocaine was administered intraperitoneally (20 mg kg^{-1}) and further nine samples were collected. Cocaine-naive animals were used for this test (Mt: $n=8$; WT: $n=8$; Ht: $n=8$; for more details: see Supplementary Information).

c-Fos activation after cocaine treatment

Cocaine-naive animals were used for this test (Mt: $n=8$; Ht: $n=7$, WT: $n=8$). Animals were transferred from the homecage to a temporary cage and injected with either saline or cocaine (15 mg kg^{-1}, intraperitoneally). Mice were left undisturbed for 70 min after injection. In line with previously established protocols[25] and to coincide with the peak neurotransmitter response seen *in vivo*, c-Fos activity was measured 70 min after cocaine administration. Thereafter, mice were culled under isoflurane narcosis and transcardially perfused. Brains were taken and c-Fos activation was measured in the NAcc (for details: see Supplementary Information).[25]

Genetic association in two independent samples of cocaine users

Initially, a total of 1459 individuals (688 cocaine/crack users and 754 healthy controls) were examined. All controls with mean age of 31.4 (±9.8) and cocaine abusers with mean age of 26.7 (±7.1) were recruited from the Blood Transfusion Unit of the Hospital das Clinicas, Faculty of Medicine, University of São Paulo and from seven drug-dependence treatment clinics in São Paulo, Brazil, respectively. The characteristics of this sample were detailed previously elsewhere.[29] Cocaine consumption was determined by a structured interview to evaluate cocaine use and risk behavior.[30] We selected 12 SNPs that cover the entire CAMK2A gene region. SNP genotyping was performed by Prevention Genetics, Marshfield, WI, USA. Individual SNPs were removed on the basis of the following criteria: call rate $<95\%$, minor allele frequency <0.01, significant deviation from Hardy–Weinberg equilibrium $P<0.05$, which led to the removal of three SNPs. We used linear regression to assess associations of remaining CAMK2A SNPs with cocaine consumption vs healthy controls and in the group of cocaine users with an index for the time to establish cocaine dependence from initial exposure to present day: $Kt=C/\Delta T$; with C: current consumption score of powder or crack cocaine (1–4; with 1 = low consumption, 4 = very high consumption);[29] ΔT: current age minus age at onset of consumption. Accordingly, higher the Kt, faster the transition to severe cocaine consumption was made. We used the PLINK v.1.07 software (http://pngu.mgh.harvard.edu/~purcell/plink/) for genetic association analyses including filtering.[31] Haplotype blocks were defined accordingly.[32] We used the Bonferroni correction procedure for multiple comparisons and selected SNPs for significant association with *P*-value <0.006. To replicate the gene effect found in this discovery sample, we analyzed three SNPs showing the strongest association effect sizes (Supplementary Table 1) in a second and independent sample of regular cocaine users from Switzerland. We genotyped the three CAMK2A SNPs (rs4958469, rs3776823, rs6881743) in 141 recreational and dependent cocaine users from the Zurich Cocaine Cognition Study, which has been described in detail before.[33–35] The isolation of the DNA from blood followed the QIAGEN protocol for the Blood & Cell Culture DNA Maxi Kit (Qiagen, Hilden, Germany). PCR was performed using 12.5 ng of DNA. The CAMK2A SNPs were analyzed by TaqMan assays (Applied Biosystems, Darmstadt, Germany). In the Swiss sample, current severity of cocaine use was determined by 6-month hair toxicologies (6 cm), in which the concentration of cocaine and of three cocaine metabolites benzoylecgonine, norcocaine and ethylcocaine was measured as described before.[33–35] Accordingly, Kt_{hair} was calculated: $Kt_{hair}=C_{hair}/\Delta T$; with C_{hair}: current cocaine hair concentration in ng mg^{-1}; ΔT = current age minus age at onset of consumption. One-way analyses of variances (ANOVAs) were used for genetic association analyses. To integrate the association results of both samples in meta-analyses, we used Stouffer's z-trend, which considers *P*-values, sample sizes and effect directions[36] and a random-effects model in which effect sizes are combined.[37]

Statistical analysis

All quantitative data were expressed as mean ± s.e.m. Given the differences in basal transmitter levels between groups, cocaine-induced neurochemical effects were expressed as a percentage of the mean of the three baseline samples, which were taken as 100% to reduce within group variability and to increase the power of the statistical test.[38] Data were analyzed using ANOVAs (for repeated measures where appropriate) followed by pre-planned comparisons using Fisher's LSD tests with Bonferroni correction when appropriate.[39] For comparisons vs baseline and for c-fos analysis, *t*-tests were used. For experiment-specific details see Supplementary Information. The software SPSS 17.0 (IBM, Armonk, NY, USA), PLINK v1.07 and Statistica 9 were used. A significance level of $P<0.05$ was set.

RESULTS

Establishment of cocaine place preference is impaired in αCaMKII^{T286A} mice

To test a potential involvement of αCaMKII autophosphorylation in the establishment of cocaine use-related behavior, we measured the time course of CPP establishment. We found that αCaMKII^{T286A}

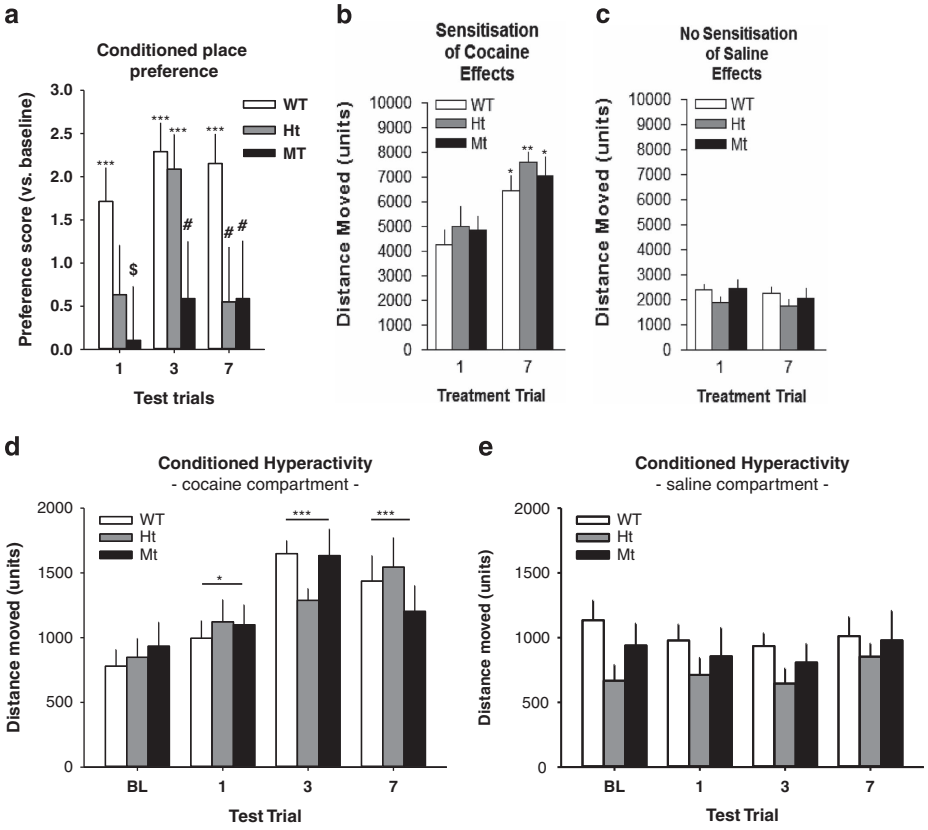

Figure 1. The establishment of conditioned place preference is impaired in αCaMKII autophosphorylation-deficient mice. (**a**) Preference vs baseline in the conditioning (cocaine-paired) compartment during the 20-min test trials (mean±s.e.m.; test trials 1, 3 and 7—after respective number of conditioning trials, Mt—αCaMKII^{T286A}, Ht—heterozygous, WT—wildtype; ***$P < 0.001$ vs baseline; $^{\$}P < 0.01$, $^{\#}P < 0.001$ vs WT). αCaMKII autophosphorylation does not affect the establishment of locomotor sensitization and conditioned hyperactivity after repeated cocaine administration (mean±s.e.m.). (**b**) Locomotor activity in the cocaine-conditioning compartment after first and seventh cocaine treatment ($^{*}P < 0.05$, $^{**}P < 0.01$, vs trial 1). (**c**) Locomotor activity in the saline-pseudo-conditioning compartment after first and seventh saline treatment. (**d**) Locomotor activity in the cocaine-conditioning compartment during test trials (BL—baseline, test trials 1, 3 and 7—after respective number of conditioning trials, two-way analysis of variance, factor time, vs BL, $^{*}P < 0.05$, ***$P < 0.001$). (**e**) Locomotor activity in the saline-pseudo-conditioning compartment during test trials (for more statistical details: see text).

mice displayed a significantly attenuated CPP establishment than WT or Ht mice. A two-way ANOVA for CPP establishment revealed a significant effect of the genotype ($F_{2,41} = 3.64$, $P = 0.035$) and test trial ($F_{2,82} = 3.26$, $P = 0.043$), but no interaction ($P > 0.05$; Figure 1a). In the WT group there was a significant CPP observed vs baseline after one ($t = 3.07$, $P = 0.009$), three ($t = 5.56$, $P < 0.0001$) and seven conditioning trials ($t = 4.57$, $P = 0.0005$). Ht mice also showed a CPP, which became significant only after three conditioning trials ($t = 4.92$, $P = 0.0002$). αCaMKII^{T286A} mice showed a reduced CPP for the cocaine-paired compartment ($P > 0.05$ vs baseline). Compared with WT, αCaMKII^{T286A} mice showed a significantly lower CPP after one ($P = 0.002$), three ($P = 0.0002$) and seven pairings ($P < 0.0001$). Ht mice CPP was only after seven pairings reduced compared with WT mice ($P < 0.0001$).

We found no evidence for a role of αCaMKII autophosphorylation in acute cocaine-induced hyperlocomotion, sensitization of hyperlocomotion (Figures 1b and c) or conditioned hyperlocomotion (Figures 1d and e; for statistical details: see Supplementary Information).

Attenuated DA responses to cocaine in αCaMKII autophosphorylation-deficient mice

αCaMKII autophosphorylation deficiency led to adaptations in basal DA and 5-HT levels (Figure 2; for statistical details: see Supplementary Information). Cocaine typically leads to an acute increase in extracellular DA levels in the NAcc and PFC of freely moving animals.[40] This was also observed in the present study in WT mice. In αCaMKII^{T286A} mice, the DA increase in the NAcc was blunted and in its peak delayed. In Ht mice, the DA response was completely absent (Figure 3a). WT animals showed a significant DA increase vs baseline 60 and 160 min after injection ($P = 0.02$ and $P = 0.03$). In Ht mice, there was a small but significant decrease vs baseline 140 min after injection ($P = 0.02$). In αCaMKII^{T286A} mice, the DA increase was statistically significant vs baseline in all intervals measured after the injection (20–40 min and 100–180 min: $P < 0.05$; 60–80 min: $P < 0.01$). A two-way ANOVA revealed significant effects of time ($F_{11,165} = 2.04$, $P = 0.03$) and genotype ($F_{2,15} = 6.41$, $P = 0.01$), and a significant interaction ($F_{22,165} = 2.12$, $P = 0.004$). Planned comparisons revealed a difference between αCaMKII^{T286A} and Ht mice 40 min ($P = 0.013$), 80 min ($P = 0.01$), 100 min ($P = 0.01$), 120 min ($P = 0.003$) and 140 min ($P = 0.03$) after cocaine injection.

In αCaMKII^{T286A} and Ht mice, the DA increase in the PFC was attenuated (Figure 3c). Extracellular DA levels increased following cocaine treatment and peaked 40 min after cocaine injection in all the groups. WT animals showed a significant DA increase vs baseline 20 min ($P = 0.02$), 40 min ($P = 0.003$), 60 min ($P = 0.0002$) and 80 min ($P = 0.02$) after injection. In Ht mice there was a significant increase vs baseline 40 min after injection ($P = 0.05$). In αCaMKII^{T286A} mice, the increase was not statistically significant ($P > 0.05$). A two-way ANOVA revealed an effect of time ($F_{11,110} = 8.61$, $P < 0.001$) and genotype ($F_{2,10} = 4.42$, $P = 0.04$), but no interaction ($P > 0.05$). Planned comparisons revealed a

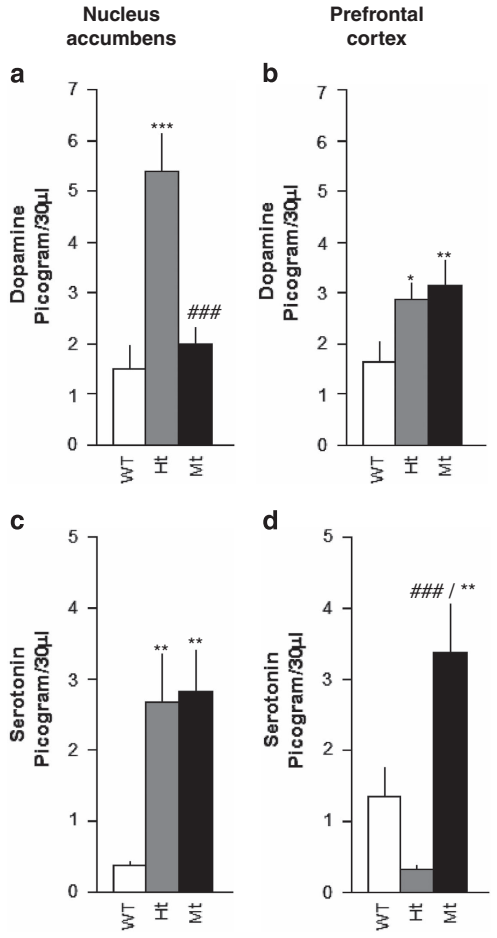

Figure 2. Baseline extracellular monoamine levels in the nucleus accumbens and prefrontal cortex (mean+s.e.m.). (**a** and **b**) dopamine, (**c** and **d**) serotonin, (Mt—αCaMKII^{T286A}, Ht—heterozygous, WT—wildtype; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs WT; $^{\#\#\#}P < 0.001$ vs Ht).

difference between WT and Ht 60 min after cocaine injection ($P = 0.015$).

Eliminated 5-HT responses to cocaine in αCaMKII autophosphorylation-deficient mice

The cocaine-induced 5-HT increase is essential for the establishment of cocaine CPP.[41] 5-HT levels were increased in response to cocaine in the NAcc in this study in WT mice (Figure 3b). This effect was absent in αCaMKII^{T286A} and Ht mice. WT animals showed a significant 5-HT increase vs baseline 20 min ($P = 0.03$) after injection. Neither Ht nor αCaMKII^{T286A} mice showed a significant change in 5-HT levels ($P > 0.05$). A two-way ANOVA revealed a significant effect of time ($F_{11,176} = 1.99$, $P = 0.03$), but not genotype effect or interaction ($P > 0.05$). Planned comparisons revealed a significant difference between Ht and WT animals 20 min after injection ($P < 0.05$).

A similar effect was observed for 5-HT in the PFC (Figure 3d). WT animals showed a tendency for a 5-HT increase vs baseline after 60 min ($P = 0.06$), followed by a small decrease 100 min ($P = 0.005$) after injection. In Ht mice there was a significant decrease vs baseline observed 20 min after injection ($P = 0.02$). In αCaMKII^{T286A} mice, 5-HT levels were decreased vs baseline 100 min ($P = 0.004$), 160 ($P = 0.02$) and 180 min ($P < 0.0001$) after injection. Although a two-way ANOVA did not show significant effects ($P > 0.05$), planned comparisons revealed a significant difference between αCaMKII^{T286A} and WT ($P = 0.01$) and between Ht and WT ($P < 0.05$) mice 60 min after cocaine injection. There was no major role for αCaMKII autophosphorylation in the acute NA response to cocaine (Supplementary Figure 1; for statistical details, see Supplementary Information).

Reduced c-Fos activation in the NAcc after cocaine in αCaMKII autophosphorylation-deficient mice

Cocaine induces neuronal activation in the NAcc, which is the base for long-term cellular, morphological and drug use-related behavior and plasticity.[42,43] To determine the downstream effects of a reduced monoaminergic response to cocaine in the αCaMKII autophosphorylation-deficient mice as measured by *in vivo* microdialysis, we investigated the effects of acute cocaine administration on c-Fos activation in the NAcc in a parallel design (Figure 4). WT mice showed a significant increase in c-Fos expression in the NAcc after cocaine treatment ($P < 0.01$). In Ht mice, there was a tendency for an increase, which did not reach statistical significance ($P > 0.05$). In αCaMKII^{T286A} mice, the cocaine-induced increase in c-Fos activity was much reduced and not significant vs saline treatment ($P > 0.05$).

An SNP in human CAMK2A gene predicts fast transition to severe cocaine use

While no functional gene mutation affecting the autophosphorylation site in humans is known, there are SNPs in the CAMK2A gene that may affect general activity and indirectly autophosphorylation. In the Brazilian discovery sample, the genotype distributions of all investigated SNPs were in accordance with Hardy–Weinberg expectations (HWE, $P > 0.1$). Of the nine SNPs analyzed from the CAMK2A gene, none predicted whether a person was a cocaine user or not ($P > 0.05$). However, within the population of the cocaine users, there was a significant association of Kt, the speed to establish severe cocaine consumption, and SNP rs3776823 ($P < 0.003$; Supplementary Table 1). Haplotype-based analysis of human genotype data showed that the highest risk for fast transition to severe cocaine use was transmitted by the presence of two T-alleles: CC = CT < TT (Figure 5a and Supplementary Figure 2).

In the Swiss replication sample, three of the CAMK2A SNPs were genotyped, which have shown the strongest associations with Kt in the Brazilian sample (rs4958469, rs3776823, rs6881743). Again, the genotype distributions of these SNPs were in accordance with HWE ($P > 0.25$). In this sample, Kt_{hair} was significantly associated with SNP rs6881743 (CC, $n = 73$: mean $Kt_{hair} = 0.91$ s.e.m. $\pm$ 0.54; CT, $n = 53$: $Kt_{hair} = 1.72 \pm 0.27$; TT, $n = 13$: $Kt_{hair} = 0.55 \pm 0.23$), whereas the genotype effect for rs3776823 was not significant (Supplementary Table 2). However, SNP rs3776823 showed the exact same pattern regarding Kt_{hair} as for Kt in the Brazilian sample (CC = CT < TT, Figure 5b) and meta-analyses of all three SNPs, considering *P*-values, sample sizes and effect directions, confirmed that only the rs3776823 SNP showed a robust and significant overall genotype effect on Kt measures across both samples (Stouffer's z-trend: $P = 0.0024$), whereas the other two SNPs were not confirmed (rs4958469, $P = 0.20$; rs6881743, $P = 0.63$). As in the Brazilian sample, homozygous carriers of the T-allele displayed a faster development to severe cocaine use than C-allele carriers (Brazilian sample Cohen's $d = 0.60$, Swiss sample $d = 0.37$). A further meta-analysis employing a random-effects model according to Hedges and Olkin[37] to combine both effect sizes of the contrast CC+CT vs TT revealed a moderate-to-strong gene effect on Kt measures (delta = 0.54, $P = 0.00008$). These findings suggest a functional effect of genetic mutations in the CAMK2A gene on how fast humans develop cocaine dependence once consumption has begun, but not on whether they become cocaine users per se.

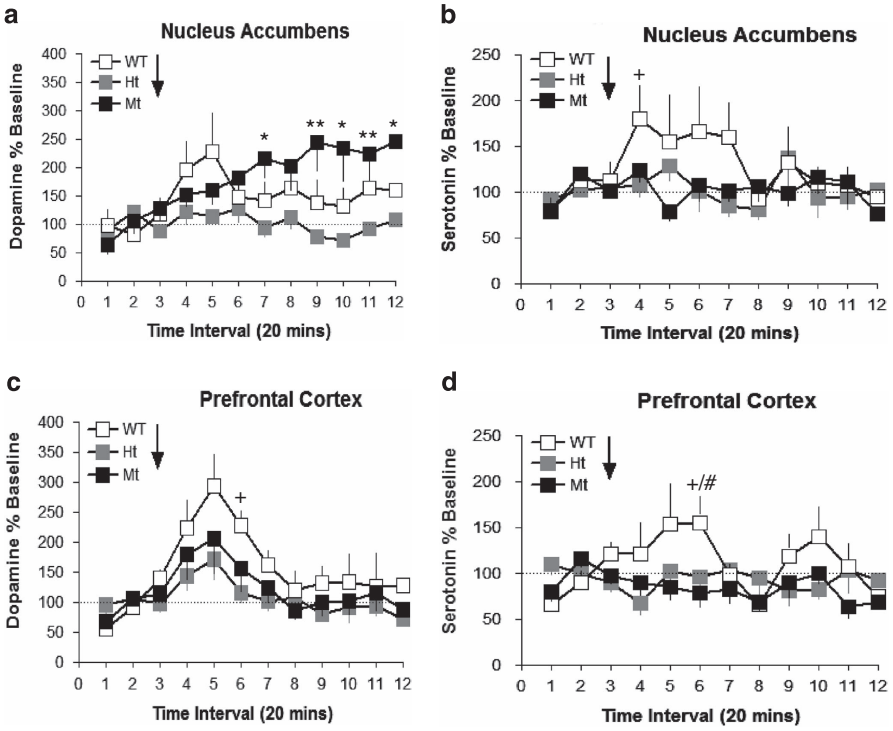

Figure 3. Acute cocaine effects on monoamine activity in the nucleus accumbens and prefrontal cortex represented as percent of baseline (mean±s.e.m.). Extracellular levels of (**a**) dopamine, and (**b**) serotonin in the nucleus accumbens after acute cocaine (20 mg kg^{-1}, intraperitoneally) treatment. Extracellular levels of (**c**) dopamine, and (**d**) serotonin in the prefrontal cortex after acute cocaine treatment. Arrows indicate time of cocaine injection (Mt—αCaMKII^{T286A}, Ht—heterozygous, WT—wildtype; $^{*}P < 0.05$, $^{**}P < 0.01$ Mt vs Ht; $^{+}P < 0.05$ WT vs Ht; $^{\#}P < 0.05$ WT vs Mt; for more statistical details, see text).

DISCUSSION

We found that αCaMKII^{T286A} mice were impaired in their ability to establish cocaine-induced CPP. αCaMKII autophosphorylation was not involved in the acute locomotor stimulant effects of cocaine, in the sensitization of hyperlocomotion or in the establishment of conditioned hyperlocomotion. The complete lack of αCaMKII autophosphorylation enhanced basal levels of monoaminergic transmitters DA and 5-HT in the NAcc and PFC, but less of NA. At the same time it attenuated the cocaine-induced DA and 5-HT increase in both brain regions. NA responses were not affected. In the NAcc, this led to a reduced cellular activation as determined by c-Fos activity. These data suggest that the reinforcing, but not locomotor stimulant effects of cocaine are under the control of αCaMKII autophosphorylation. A translational approach, using two independent human samples of cocaine-dependent individuals revealed a robust link between a polymorphism in the αCaMKII coding gene (rs3776823) and the speed of establishing severe cocaine consumption.

A major finding of this study was that αCaMKII autophosphorylation controls the establishment rate of cocaine CPP. This finding is in line with recent reports, which support a role of CaMKII in the ventral tegmental area[19] and of CaMKII autonomous activity in the NAcc shell[44] in the establishment of cocaine CPP. While CPP reflects the rewarding properties of a drug, it can be derived from a number of different behavioral processes.[45] In this study, however, we did not find differences in acute locomotor or conditioned locomotor effects of cocaine between genotypes which may explain the reduced CPP establishment in the αCaMKII^{T286A} mice. The lack of a genotype effect on locomotor activity may also rule out a reduced bioavailability or general insensitivity to cocaine in the αCaMKII^{T286A} mice. Although αCaMKII^{T286A} mice have severe problems to learn aversively motivated tasks,[20,21] they have little problems to establish positively reinforced behavior using natural reinforcers.[46] A limitation of the present study is that no compulsive self-administration paradigm could be applied in the mice. Therefore, conclusions must be limited to the role of αCaMKII autophosphorylation in controlled cocaine use, rather than in addiction. Altogether, present data may suggest that a lack of αCaMKII autophosphorylation prevented the association of the incentive properties of cocaine with environmental cues.

The DA system is critical for the establishment of the acute reinforcing effects of cocaine.[47] We have shown that an acute cocaine challenge induces a DA increase in the NAcc and PFC of WT mice. This response was entirely absent in Ht mice and blunted or attenuated in the αCaMKII^{T286A} mice. Neuroadaptive processes initiated by drugs of abuse are sensitive to the rate of drug administration.[48] It was suggested that a fast rate of administration will facilitate neurobehavioral plasticity, which in turn contributes to excessive drug use.[49] The slower initiation of the DA response in αCaMKII^{T286A} mice could, therefore, account for the delay in cocaine-induced CPP. The present finding suggests that αCaMKII autophosphorylation is required for the cocaine-induced DA increase in the NAcc. However, we also observed increased basal DA levels in the PFC of αCaMKII^{T286A} mice. Transmitter levels before cocaine treatment may reflect a complex role for αCaMKII autophosphorylation in the resting state of the reward system, thus influencing response to cocaine administration and rate of transmitter release. CaMKII can phosphorylate a number of intracellular targets[50] including AMPA receptors,[51] NMDA receptors,[50] L-type Ca^{2+} channels[52] and tyrosine hydroxylase[53] the rate-limiting enzyme in DA synthesis. Furthermore, tyrosine hydroxylase, DAT, calmodulin and CaMKII are co-distributed in mesolimbic projections,[54] and it has been suggested that DA synthesis is regulated by CaMKII-controlled tyrosine hydroxylase activity.[55] Thus, the increase in basal DA levels may, in our study, have limited the capacity for further DA increase in the PFC.

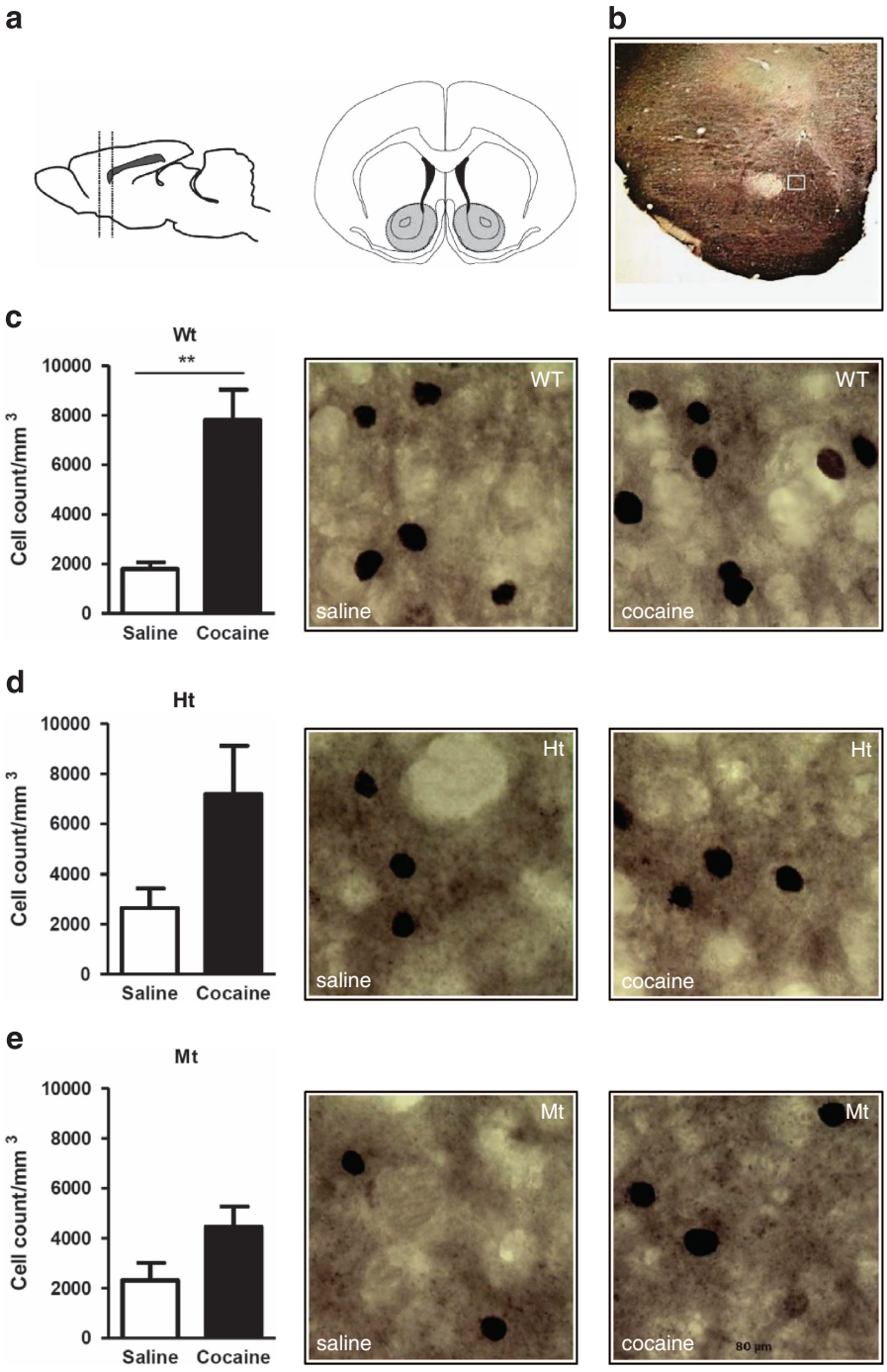

Figure 4. αCaMKII autophosphorylation-deficient mice show a reduced increase in c-Fos expression after single cocaine (15 mg kg^{-1}, intraperitoneal) treatment in the nucleus accumbens (mean±s.e.m.). (**a**) Localization of the scored c-Fos activation in the brain (white square). (**b**) Low amplification image of the localization of sample photomicrographs. c-Fos labeling in (**c**) wildtype (WT), (**d**) heterozygous (Ht) and (**e**) αCaMKII^{T286A} (Mt) mice ($^{**}P < 0.01$). Photomicrographs show c-Fos-labeled cells (black) of the nucleus accumbens (scale: 80 × 80 μm).

Previous work by Sora *et al*.[56] using monoamine transporter knockout mice showed that neither DAT nor SERT deletion alone can block the establishment of cocaine CPP, but that concurrent deletion of both, DAT and SERT, can prevent it. A major role of 5-HT in the establishment of cocaine CPP is also supported by lesioning approaches.[41] Cocaine induces a robust 5-HT increase in the mesocorticolimbic system,[57–60] which was also observed in this study. The 5-HT increase after cocaine in the NAcc and PFC was abolished in the αCaMKII^{T286A} and Ht mice. However, basal 5-HT levels were significantly enhanced in these animals before treatment. Tryptophan hydroxylase 2 (TPH2) is the initial and rate-limiting enzyme in the biosynthesis of 5-HT in the brain and can be activated by CaMKII.[61] Thus, alterations in the activity of CaMKII, and consequently activity of TRP2, could ultimately influence the function of serotonergic nerve endings by increasing or decreasing the amount of transmitter available for release into the extracellular space. This might suggest a limiting capacity for cocaine-induced increase in extracellular 5-HT activity in αCaMKII^{T286A} mice. Absence of a cocaine-induced 5-HT response in αCaMKII^{T286A} mice may have contributed towards the observed

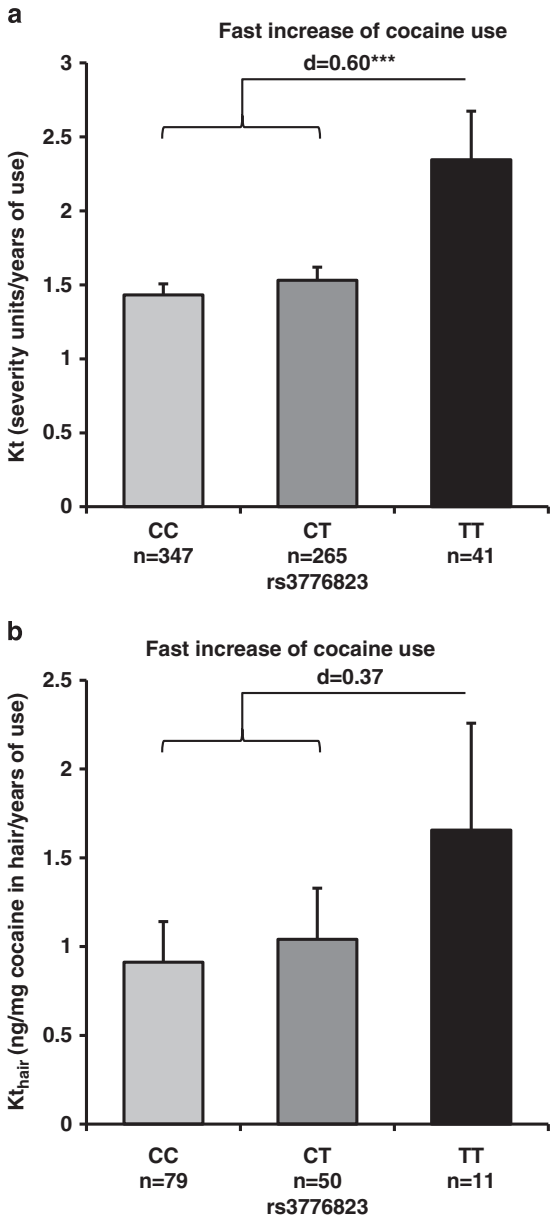

Figure 5. A human CAMK2A gene polymorphism predicts fast transition to severe cocaine use. (**a**) Significant associations of the CAMK2A polymorphism, rs3776823, with the fast transition to severe cocaine use in a Brazilian population of cocaine-dependent individuals (mean±s.e.m.). Kt is an index for the time to establish severe cocaine consumption from initial exposure to present day. $Kt = C/\Delta T$; with C: current consumption score of powder or crack cocaine (1–4; with 1 = low consumption, 4 = very high consumption). Homozygous T-allele carriers displayed significantly higher Kt scores than C-allele carrieres reflecting a faster transition to severe cocaine consumption (***$P < 0.001$, analysis of variance (ANOVA), TT vs CC+CT). (**b**) Associations of the CAMK2A polymorphism, rs3776823, with the fast transition to severe cocaine consumption in a Swiss population of recreational and dependent cocaine users (mean±s.e.m.). Here, Kt_{hair} was calculated using cocaine hair concentrations as a severity marker: $Kt_{hair} = C_{hair}/\Delta T$; with C_{hair}: current cocaine hair concentration in ng mg^{-1}; ΔT = current age minus age at onset of consumption. Similar to the Brazilian sample, homozygous T-allele carriers revealed higher Kt_{hair} scores than C-allele carriers, however, the difference was not significant due to a much smaller sample size but showed a considerable effect size (ANOVA TT vs CC+CT: $P = 0.27$, $d = 0.37$).

CPP deficit. Evidence implicates CaMKII in the regulation of DA, 5-HT and NA synthesis in the central nervous system, suggesting that CaMKII may act as a mediator for basal and cocaine-induced activity for all three transmitters. In addition, we cannot rule out alterations in reuptake mechanisms and metabolic enzymes as a result of this mutation, or indeed cocaine administration itself, as having a crucial impact on extracellular transmitter levels seen in the present study. Such alterations, namely cocaine blockade of the DA transporter, may go some way towards explaining the notable DA increases in the NAcc of Mt mice, which persist 120+ minutes after cocaine administration, although we cannot definitively say this using current data alone and further investigation would be required.

Cocaine effects on NAcc synaptic plasticity appeared to be under control of CaMKII in the ventral tegmental area,[19] which may, as the present study suggests, be transmitted to the NAcc by activity modulated dopaminergic projections. A crucial indicator of neuronal activation after a drug challenge is the immediate early gene c-Fos,[61] which becomes activated in the NAcc and other brain areas after acute cocaine administration by a DA and 5-HT dependent mechanism.[62] We found reduced c-Fos activation in the NAcc of $\alpha CaMKII^{T286A}$ and Ht mice. This is in line with the attenuated monoamine response and suggests an attenuated neuronal activation in the NAcc following cocaine administration, which may explain the delayed establishment of cocaine's reinforcing effects.

Cocaine alters αCaMKII expression in the human brain.[14] Here we report an association between an SNP in the human CAMK2A gene and the time to establish severe cocaine consumption in human regular cocaine users. The identified SNP, rs3776823, is an intronic SNP, but only 425 bp upstream of exon 4 of the CAMK2A gene. Exon 4 codes for the amino acids 73–92 of αCaMKII. This is a substrate-binding pocket of the catalytic domain of αCaMKII. Furthermore, this SNP may regulate splice factor SC35 binding and thus exon 4 splicing. These findings suggest that polymorphisms in the CAMK2A gene may contribute to the speed of acquiring a severe level of regular cocaine use once consumption has commenced. Although there was a genetic overlap, that is, jointly associated gene polymorphisms, in the associations between alcohol addiction and learning and memory,[9,25] no such overlap was found with the speed to establish severe cocaine use, which might suggest different mechanisms. A limitation of the human genetic study is clearly that we could not determine polymorphisms associated with αCaMKII autophosphorylation in humans, but only with αCaMKII function in general. Human findings may therefore relate to Ca^{2+}-dependent or Ca^{2+}-independent αCaMKII activation.

In summary, the present study suggests that αCaMKII might have an important role in the speed to establish cocaine's reinforcing action in mice and humans by controlling monoaminergic activation and downstream neuronal activity in the reward system.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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